# NTP Research Concept: Isoflavones in Soy Infant Formula

## Project Leader

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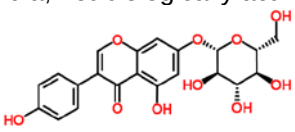
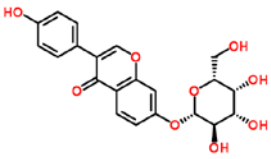
### Figure 1. Isoflavones associated with soy infant formula

**Sugar-bound isoflavones**
(present in soy infant formula, not biologically active)

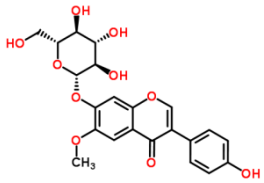
**Genistin**
$C_{21}H_{20}O_{10}$
MW: 432.37
CAS: 529-59-9



**Daidzin**
$C_{21}H_{20}O_9$
MW: 416.37
CAS: 552-66-9

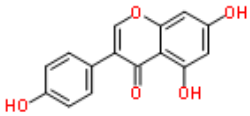


**Glycitin**
$C_{22}H_{22}O_{10}$
MW: 446.41
CAS: 40246-10-4

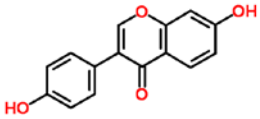


**Aglycone isoflavones**
(biologically active)

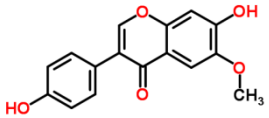
**Genistein**
$C_{15}H_{10}O_5$
MW: 270.24
CAS: 446-72-0



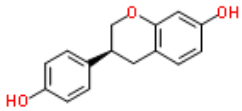
**Daidzein**
$C_{15}H_{10}O_4$
MW: 254.24
CAS: 486-66-8



**Glycitein**
$C_{16}H_{12}O_5$
MW: 284.26
CAS: 40957-83-3



**Equol***
$C_{15}H_{14}O_3$
MW: 242.27
CAS: 531-95-3



*Daidzein can be metabolized to equol in some humans and experimental animals.

## Background and Rationale

Soy infant formula contains soy protein isolate and is fed to infants as a supplement to or a replacement for human milk, or as an alternative to cow milk formula. Soy protein isolate contains isoflavones with estrogenic activity called “phytoestrogens,” a subset of plant-derived compounds with biological activity similar to the female hormone estrogen. The three major phytoestrogens found in soy infant formula are predominantly the sugar-bound forms: genistin, daidzin and glycitin. These phytoestrogens are biologically active in their aglycone form (sugar-free and unconjugated): genistein, daidzein, and glycitein, respectively. The relative abundance of the isoflavones in soy infant formula (expressed in aglycone units) is: genistein (58-66%) > daidzein (29-34%) > and glycitein (5-8%). In some humans, daidzein can be metabolized to another estrogenic metabolite called equol (Figure 1). The relative estrogenic activity of the isoflavones based on *in vitro* studies is genistein > equol > daidzein > glycitein.

In October 2008, the NTP initiated an updated evaluation of soy infant formula. The evaluation process used to help develop the conclusions presented in the draft NTP Brief included convening a panel of scientific experts to produce a report on: (1) the

strength of the scientific evidence that soy infant formula or its isoflavone constituents are developmental toxicants based on data from *in vitro*, animal, or human studies; (2) the extent of isoflavone exposures in infants fed soy infant formula; (3) the assessment of the scientific evidence that adverse developmental health effects may be associated with such exposures; and (4) the identification of data gaps in the soy infant formula and isoflavone literature to reduce uncertainties and increase confidence in future evaluations. The final expert panel report was released for comments on January 15, 2010 (http://cerhr.niehs.nih.gov/chemicals/genistein-soy/SoyFormulaUpdt/FinalEPReport_508.pdf).

On March 16, 2010, the NTP released its draft Brief on Soy Infant Formula, which expresses "minimal concern" for adverse effects on human development due to the consumption of soy infant formula (available at http://cerhr.niehs.nih.gov/chemicals/genistein-soy/soyformula/soyformula-eval.html). The draft NTP conclusion of "minimal concern" was based primarily on laboratory animal toxicity studies of genistein and the extent of human exposure to genistein in infants who are fed soy formula:

- There was "clear evidence" for adverse effects of genistein on reproductive development and function in female mice treated only during lactation and in female rats in an NTP multigenerational study of genistein. Effects were manifested as accelerated puberty (i.e., decreased age at vaginal opening), abnormal estrous cyclicity, cellular changes to the female reproductive tract , and decreased fecundity (i.e., decreased fertility, implants, and litter size) (Jefferson *et al.* 2009; Jefferson *et al.* 2006; Jefferson *et al.* 2002; Jefferson *et al.* 2005; Newbold *et al.* 2001; NTP 2008; Padilla-Banks *et al.* 2006). Mammary gland hyperplasia was also observed in male rats with genistein exposure during pregnancy and lactation, and beyond weaning in an NTP multigenerational study in rats at dietary dose levels below those where clear adverse effects on the female reproductive tract were observed (Delclos *et al.* 2001; NTP 2008). In contrast, there was "insufficient evidence" to reach a conclusion for adverse developmental effects in laboratory animals based on studies of soy infant formula, soy protein isolate, soy-based diets, other individual isoflavones (daidzein, glycitein, or equol), or mixtures of isoflavones.

- Although there is a relatively large literature describing growth or other health parameters in infants fed soy infant formula, this literature was considered "insufficient evidence" to reach a conclusion on whether the use of soy infant formula adversely affects human development with respect to effects on bone mineral density, allergy/immunology, thyroid function, reproductive system endpoints, cholesterol, diabetes mellitus, and cognitive function. However, the blood levels of genistein in infants fed soy infant formula can exceed those measured in neonatal or weanling rodents following treatment with genistein at dose levels that induced adverse effects in the animals (Cao *et al.* 2009; Chang *et al.* 2000; Doerge *et al.* 2002).

**Data gaps identified by the NTP CERHR Expert Panel on Soy Infant Formula**

The expert panel identified a number of data gaps and research needs in the current literature in laboratory animals that limited its utility for reaching conclusions for infants fed soy infant formula. The following three issues summarize some of these data gaps.

1. The first issue is the differences between the period of exposure utilized in the animal studies and the relevant period of human exposure to soy infant formula. Many of the animal studies considered in the NTP evaluation of soy infant formula included isoflavone exposure during the period of gestation, lactation, and beyond weaning, which made it difficult to distinguish the effects that might have occurred as a result of exposure during lactation alone. Thus, the NTP multigenerational study of genistein, where clear evidence of developmental toxicity in females was observed, was characterized as "limited" utility by the expert panel. Furthermore, there is limited lactational transfer of genistein to rat pups when the dam is directly treated (Doerge *et al.* 2006). Data from young animals exposed directly to soy infant formula or isoflavones during the period of lactation only would provide a better approximation of human exposure of infants fed soy infant formula.

2. The second issue is the potential interaction of mixtures of isoflavones present in soy infant formula and its impact on the toxicity of these isoflavones. In particular, the laboratory animal studies that supported "clear evidence" for adverse effects on reproductive development and function involved administration of genistein only and therefore do not address the potential for interaction between other isoflavones or non-isoflavone components found in soy infant formula to impact the effects observed after exposure to genistein only. It is unknown if the other estrogenic isoflavones present in soy infant formula (i.e.,daidzein, glycitein, and equol) act in a dose-additive fashion or if there is antagonism between the isoflavones in soy infant formula that may impact the effects of genistein alone.

3. A third issue is related to daidzein exposure and the degree to which its estrogenic metabolite equol might contribute to the *in vivo* estrogenic activity of daidzein. Only 30-50% of adult humans are equol producers. Human infants are generally considered less able to produce equol from daidzein compared to adults due to immaturity of gut microflora and/or underdeveloped metabolic capacity. In contrast, adult rodents (rats, mice, and guinea pigs), and primates used as animal models are reported to produce equol. The developmental profile of equol production is unknown in rats and mice, the main species used to characterize isoflavone toxicity. Understanding differences in comparative physiology can aid in understanding the estrogenic effects of daidzein across species.

Thus, the NTP is proposing to conduct a series of studies that will address data gaps and reduce these areas of uncertainty for reaching conclusions on human infants fed soy infant formula from the laboratory animal data. The two major goals of this research effort are: (1) to determine whether lactation-only exposure would result in the adverse effects described above from NTP multigenerational studies and other studies of

isoflavones where exposure encompassed a period greater than the period of lactation, and (2) to determine whether the findings described above that support "clear evidence" of adverse effects from genistein-only studies are also observed with isoflavone mixtures present in soy formula.

## Specific Aims

1. Determine the effects of lactation-only treatment with soy infant formula on the reproductive development and fertility of rodents (rats and/or mice) by oral dosing to the pups. The NTP will explore the feasibility of administering soy infant formula during lactation-only to rodent pups in a reproductive development and fertility study because it would be the best model for human infants fed soy infant formula. In addition, the administration of soy infant formula to rodent pups would test for all components of soy infant formula, including the non-isoflavones components.

2. Determine the effects of lactation-only treatment with a mixture of isoflavones at the ratio found in soy infant formula on the reproductive development and fertility of rodents (rats and/or mice) by oral dosing to the pups.

    a. Determine whether the published findings described above that support "clear evidence" of adverse effects from genistein-only studies are also observed with an isoflavone mixture of genistin, daidzin, and glycitin.

    b. Determine whether effects from lactation-only treatment with a mixture of isoflavones differ from exposure that includes the non-isoflavone content of soy infant formula (i.e., are results the same or different from any results of the soy infant formula exposure described in "1").

3. Determine how the individual isoflavones in soy infant formula interact with one another in a short term estrogenic bioassay.

    a. Determine whether the isoflavones exert a cumulative, dose additive effect on the estrogen-dependent uterotrophic assay.

    b. Evaluate whether the less potent isoflavones in soy infant formula may antagonize the effects of genistein on the estrogen-dependent uterotrophic assay.

4. Determine the developmental profile of daidzein metabolism to equol during postnatal development in mice or rats by measuring blood levels of total and aglycone daidzein and equol, following oral exposure of daidzin.

## Proposed Approach

1. **Studies to determine the feasibility of direct oral administration of soy infant formula to rodents (rats and/or mice) during the period of lactation (Specific Aim 1).**

Administering soy infant formula to rodent pups to achieve isoflavone levels comparable to human infants fed soy formula is challenging. Therefore, the NTP will explore the feasibility of administering rodent pups soy infant formula in a concentrated form and via multiple doses per day prior to implementing a study evaluating the effects of soy infant formula on reproductive development and fertility.

2. **Reproductive development and fertility study in rodents (rats and/or mice**). **(Specific Aims 1 and 2)**

These studies will test whether lactation-only exposure of rodent pups to soy infant formula or an isoflavone mixture of genistin, daidzin or glycitin (in the ratio present in soy infant formula) induces adverse effects on reproductive development and fertility. Rodent pups will be administered the treatments via direct oral dosing. The sugar-bound forms of genistein, daidzein and glycitein will be administered in all of the proposed studies because they are the predominant forms of these isoflavones in soy infant formula. These studies may be conducted in either rats and/or mice. The mouse model would be useful to compare to the effects observed in female mice exposed to genistein or genistin during of the first five days of lactation (PND1-5) (Jefferson *et al.* 2009; Jefferson *et al.* 2006; Jefferson *et al.* 2002; Jefferson *et al.* 2005; Newbold *et al.* 2001; Padilla-Banks *et al.* 2006). The rat model would be useful to compare the effects seen in both female and male rats exposed to genistein via their dam during gestation and lactation as well as beyond weaning (NTP 2008). In addition, male mice do not have detectable mammary gland tissue, while mammary gland hyperplasia occurred in male rats at lower dose levels of genistein than did the female effects (NTP 2008). Appropriate pharmacokinetic studies would be conducted to determine the blood levels of total and aglycone isoflavones, including testing for equol.

3. **Uterotrophic assay in rodents (rats and/or mice) (Specific Aim 3).**

The purpose of this study is to use a short-term *in vivo* assay as an indicator of the estrogenic potential of the predominant isoflavones present in soy infant formula (i.e., genistin, daidzin, and glycitin) and how they interact as a mixture on estrogen-responsive development. The isoflavone mixtures are predicted to stimulate estrogen-responsive uterine wet weight in the neonatal uterotropic assay in a cumulative and additive manner, based on the relative *in vitro* estrogenic activity of the isoflavones (genistein > equol > daidzein >> glycitein) (e.g., Choi et al. 2008). These studies will also evaluate the alternate hypothesis that the less potent isoflavones may act as estrogen receptor antagonists inhibiting the activity of genistein, thus limiting estrogenic effects on reproductive tract development.

The neonatal mouse uterotrophic assay is responsive to genistein and genistin exposure (Jefferson *et al.* 2009) and would allow for an assessment of the estrogenic response during the period of lactation, which is the relevant period of exposure to soy infant formula. However, the rat weanling uterotrophic assay will be used as an alternative short-term estrogenic assay, if the mouse neonatal assay is too variable or does not produce a sufficient magnitude of uterine stimulation for analyses of isoflavone mixture effects or evidence of antagonism.

4. **Pharmacokinetic studies to assess metabolism of daidzin to equol during the period of lactation (Specific Aim 4)**

The NTP will evaluate whether equol is being produced during the period of lactation. Mouse pups will be exposed orally to daidzin during the period of lactation from PND1-5. If equol is not detected on PND5, then additional pharmacokinetic studies will be conducted to determine onset of equol production during later periods of lactation. If the rat model is used to address Specific Aims 1, 2 and/or 3, pharmacokinetic studies may be similarly employed to evaluate the developmental profile of equol production in the rat.

## Significance and Expected Outcomes

The proposed NTP studies address several of the key data gaps identified by the NTP Expert Panel on Soy Infant Formula. The proposed NTP studies will provide data on the possible effects of soy infant formula on reproductive development and fertility in rodents with a focus on soy infant formula (if feasible) and the isoflavone components of soy infant formula. The lactation-only exposure studies will provide animal data that is restricted to the most relevant period of human infant exposure to soy infant formula as identified by the NTP CERHR Expert Panel. The results of the lactation-only exposure studies can be used to compare and contrast with the existing database of reproductive development and fertility studies that have evaluated the effects of isoflavone exposure that were not restricted to the period of lactation and to the published literature on individual isoflavone exposures. The rodent uterotrophic assay data will provide valuable information on how the individual isoflavones in soy infant formula interact with one another to influence estrogen-responsive tissues *in vivo*. The strength of the uterotropic assay is the use of a short term *in vivo* estrogen-dependent response to evaluate the effects of a dose range of individual isoflavones and isoflavone mixtures. Knowledge of the developmental profile of equol production in the rodent pup, following daidzin exposure, will be useful in determining whether equol is produced during the period of lactation in rodents and may explain effects not generally predicted by the potency of daidzein as determined by *in vitro* assays.

## References

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